# Human gastrin-releasing peptide receptor mediates sustained CREB phosphorylation and transactivation in HuTu 80 duodenal cancer cells

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**Abstract The G protein-coupled human gastrin-releasing peptide receptor (hGRP-R) is frequently found aberrantly expressed in human cancers of the colon, stomach, and lung, and its ligand-specific activation has been implicated in cell proliferation and differentiation. Here, we demonstrated hGRP-R activation stimulated sustained cyclic AMP response element binding protein (CREB) phosphorylation and transactivation in duodenal cancer cells through a protein kinase C and partially p38 mitogen-activated protein kinase-dependent pathway. In contrast, intracellular calcium, ERK1/2, protein kinase A, and PI3 kinase were not involved. This novel signaling mechanism might be of importance for regulation of CREB-dependent gene expression in human cancer expressing functional hGRP-R.** 

*Key words:* Gastrin-releasing peptide receptor; Cyclic AMP response element binding protein phosphorylation; Bombesin; Protein kinase C; p38 mitogen-activated protein kinase; Human cancer

## 1. Introduction

The human gastrin-releasing peptide receptor (hGRP-R) belongs to the mammalian bombesin (Bn) receptors which comprise a family of three $G_q$ protein-coupled receptors, including the GRP-R, the neuromedin B receptor, and the orphan bombesin receptor subtype 3 [1]. GRP-R mediates diverse physiological central nervous and gastrointestinal functions by binding of its specific ligand, the regulatory GRP [2]. Noticeably, hGRP-R is often found aberrantly expressed in human cancers of the colon, stomach, lung, breast, and prostate [1]. When aberrantly expressed in cancer, GRP-R has been implicated to mediate the effects of GRP primarily as a potent mitogen and as morphogen, thus having an important role in human cancer [3–5]. However, the underlying intracellular signaling mechanisms of this receptor involved in human carcinogenesis have not been fully elucidated.

In this study, we demonstrated for the first time that ligand-specific hGRP-R activation in duodenal cancer cells mediated sustained cyclic AMP response element binding protein (CREB) phosphorylation and transactivation in a protein kinase C (PKC)- and partially p38 mitogen-activated protein kinase (MAPK)-dependent, but protein kinase A (PKA)-, ERK1/2-, and calcium-independent manner. Since CREB is a critical transcription factor involved in many cellular functions [6], our results suggest the possibility that this novel signaling pathway might result in long-term regulation of CREB-dependent gene transcription relevant in human cancer cells expressing functional hGRP-R.

## 2. Materials and methods

### 2.1. Materials

Forskolin (FSK) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Bisindolylmaleimide I (GFX), SB 203580, U 0126, H-89, wortmannin, rapamycin, BAPTA/AM, and thapsigargin were purchased from Calbiochem (San Diego, CA, USA). Bn was from Calbiochem (San Diego, CA, USA). The GRP-R antagonists (3-phenylpropanolyl-D-Ala$^{24}$, Pro$^{26}$, Psi$^{26,27}$, Phe$^{27}$)GRP-(20–27) (BW2258U89) and (D-F5-Phe$^{6}$, D-Ala$^{11}$)Bn(6–13)methyl ester (ME) were kindly provided by Drs. T.W. Moody and R.T. Jensen (National Institutes of Health; Bethesda, MD, USA). Antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

### 2.2. Cells

HuTu 80 cells, a duodenal cancer cell line, were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. Prior to Bn stimulation, cells were synchronized in serum-free medium for 36 h.

### 2.3. Plasmids and transient transfections

Reporter plasmids pFR-Luc, pFA2-CREB (fusion transactivator) were purchased from Stratagene (La Jolla, CA, USA). 4×Som-CRE-Luc plasmid (containing four copies of the rat somatostatin gene CRE sequence in pT81Luc) was generously provided by Prof. Knepel (University of Göttingen, Göttingen, Germany). pCMVβ-gal expression vector was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). For transient transfections, cells were transfected with reporter plasmid and fusion transactivator plasmid using lipofectAMINE™ reagent (Life Technologies, Inc., Carlsbad, CA, USA). pCMVβ-gal plasmid was co-transfected into cells along with the reporter plasmid to assess transfection efficiency. Cells were treated with Bn and FSK for 16 h and harvested for luciferase and β-galactosidase activity assay as previously described [7].

### 2.4. Western blot analysis

Cells were lysed in sample buffer (62.5 mM Tris–HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue). Samples containing equal amounts of total protein were resolved in 10% SDS–polyacrylamide gel, transferred to nitrocellulose, incubated with the primary antibody at 4°C overnight, and finally incubated with the secondary antibody for 1 h at room

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*Abbreviations:* hGRP-R, human gastrin-releasing peptide receptor; Bn, bombesin; CREB, cyclic AMP response element binding protein; PKC, protein kinase C; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; FSK, forskolin

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temperature. The antibody–antigen complexes were then detected using ECL reagent (Amersham Pharmacia Biotech Inc.; Arlington Heights, IL, USA).

## 3. Results

### 3.1. Bn stimulates sustained CREB phosphorylation at Ser$^{133}$ via hGRP-R in HuTu 80 cells

The tetradecapeptide Bn is the amphibian homologue of the 27-amino acid mammalian GRP and possesses specific, high-affinity binding properties on the GRP-R [1]. Using a specific antibody against the phosphorylated form of CREB at Ser$^{133}$ in immunoblots, we showed that hGRP-R mediated Bn-dependent CREB phosphorylation in HuTu 80 cells in a dose-dependent manner (Fig. 1A). Bn-induced CREB phosphorylation occurred as early as 5 min after Bn stimulation (data not shown), and was sustained for at least 8 h (Fig. 1B). Since the primary structures of the CREB family all contain the same kinase-inducible domain (KID) region, the anti-phospho CREB-specific antibody (Ser$^{133}$) also detected the phosphorylated form of ATF-1. Our data showed that ATF-1 phosphorylation was induced concomitantly with Bn-induced CREB

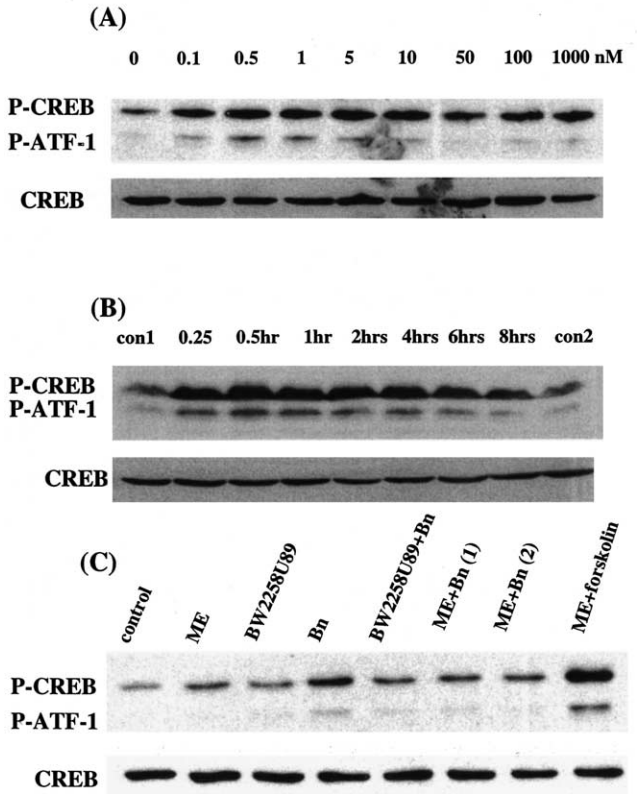


Fig. 1. CREB is phosphorylated at Ser$^{133}$ via hGRP-R activation in HuTu 80 cells. A: CREB phosphorylation (P-CREB) after Bn stimulation for 30 min occurred in a dose-dependent manner. B: Bn (10 nM) induced sustained CREB phosphorylation for at least 8 h (con1 is Bn at 0 min, con2 is vehicle control at 8 h). C: Pretreatment with hGRP-R antagonists BW2258U89 (1 μM) and ME (100 nM) for 20 min prior to stimulation with Bn (10 nM) for 30 min abolished Bn-induced CREB phosphorylation. ME+Bn (1) and ME+Bn (2) are independent duplicates of the same treatment (Bn stimulation after ME pretreatment). Both, phosphorylated CREB at Ser$^{133}$ (P-CREB) and total CREB, were detected by immunoblotting. Anti-phospho-CREB-specific antibody also detected the phosphorylated form of the CREB-related ATF-1 (P-ATF-1).

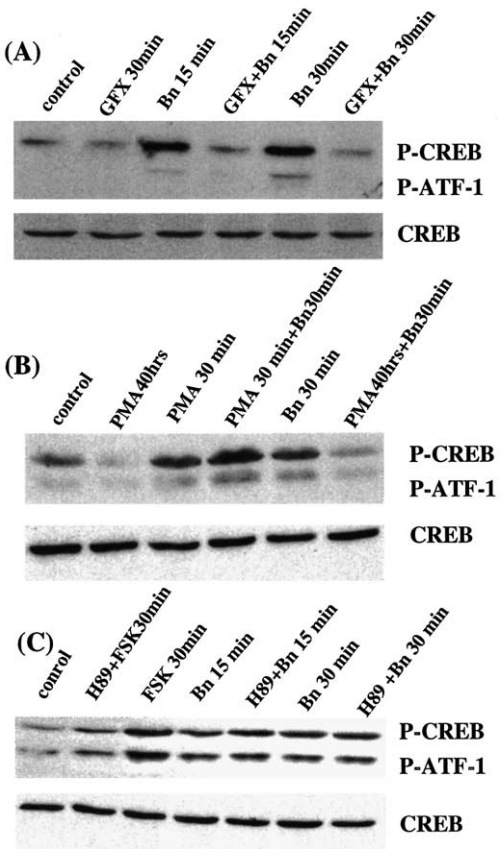


Fig. 2. Bn-induced CREB phosphorylation is PKC- but not PKA-dependent. A: Pretreatment with GFX (2 μM) prior to Bn (10 nM) stimulation abolished CREB phosphorylation completely at 15 and 30 min, respectively. B: Down-regulation of PKC with long-term PMA pretreatment (100 nM; 40 h) diminished the Bn-dependent CREB phosphorylation. Short-term incubation (30 min) with PMA alone enhanced CREB phosphorylation. C: Pretreatment with H-89 (20 μM) abolished FSK (25 μM)-induced CREB phosphorylation but had no effect on CREB phosphorylation in response to Bn stimulation for 15 and 30 min, respectively.

phosphorylation. To determine the specificity of Bn-induced CREB phosphorylation through hGRP-R activation, we used the specific GRP-R antagonists BW2258U89 and ME 20 min before stimulation with Bn. Both BW2258U89 and ME abolished Bn-induced CREB phosphorylation as shown in Fig. 1C.

### 3.2. Bn-induced CREB phosphorylation is PKC-dependent, but PKA- and calcium-independent

To investigate upstream signaling molecules, we investigated Bn-induced CREB phosphorylation in the presence of different inhibitors. Pretreatment of cells with the PKC inhibitor GFX (2 μM) completely abolished CREB phosphorylation (Fig. 2A). In contrast, GFX did not affect FSK-induced CREB phosphorylation (data not shown). Furthermore, after down-regulation of PKC by long-term pretreatment with PMA (100 nM; 40 h) Bn induced CREB phosphorylation only marginally (Fig. 2B). In contrast, treatment with PMA alone for 30 min resulted in enhanced CREB phosphorylation, and Bn stimulation following short-term PMA treatment further induced CREB phosphorylation (Fig. 2B). The PKA inhibitor H-89 (20 μM) did not affect Bn-stimulated CREB phosphorylation (Fig. 2C), whereas it almost abolished FSK-

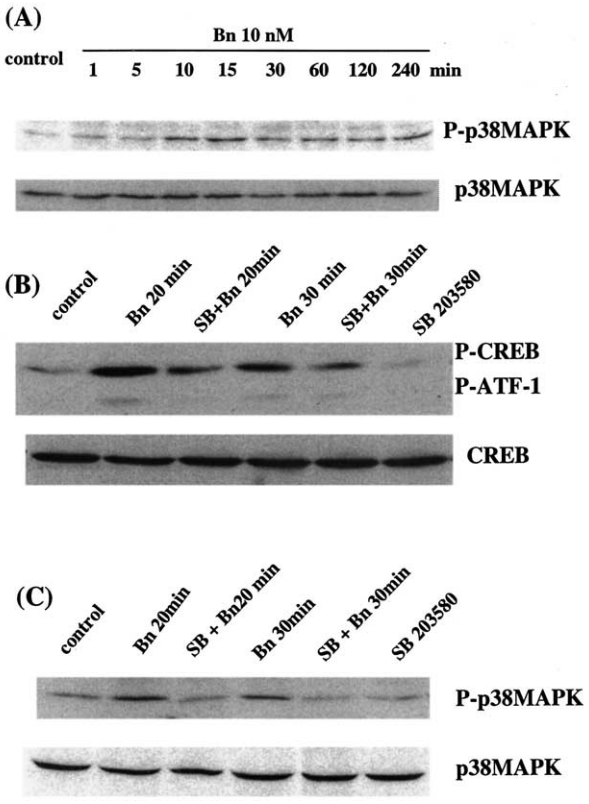


Fig. 3. p38 MAPK pathway might be partially involved in CREB phosphorylation via hGRP-R activation. A: Bn (10 nM) induced moderate sustained p38 MAPK phosphorylation for at least 4 h. B: Pretreatment with SB 203580 (20 μM) before Bn stimulation resulted in reduction of CREB phosphorylation. C: p38 MAPK phosphorylation was abolished by SB 203580 (20 μM). p38 MAPK dual phosphorylation at $Thr^{180}$ and $Tyr^{182}$ was detected by immunoblot using a phospho-specific anti-p38 MAPK antibody (P-p38MAPK).

induced CREB phosphorylation as expected. To determine whether calcium-dependent pathways are involved, BAPTA/AM (30 μM) was used to chelate intracellular $Ca^{2+}$, and thapsigargin (2 μM) was used to inhibit intracellular $Ca^{2+}$ release before Bn stimulation. We found that inhibition of intracellular calcium elevation did not yield any changes of Bn-induced CREB phosphorylation (data not shown). Similarly, inhibition of PI3 kinase signaling with wortmannin (200 nM) or inhibition of p70 S6 kinase with rapamycin (100 nM) did not affect Bn-induced CREB phosphorylation (data not shown).

### 3.3. *p38 MAPK pathway might be involved in Bn-induced CREB phosphorylation*

To further explore alternative signal mechanisms involved in Bn-dependent CREB phosphorylation, we investigated the activation of different MAPK pathways. Using a phospho-specific p38 MAPK antibody, we showed that Bn stimulated p38 MAPK phosphorylation moderately in a sustained manner (Fig. 3A). p38 MAPK phosphorylation occurred from 10 min to at least 4 h after Bn stimulation. Using the p38 MAPK inhibitor SB 203580 (20 μM) prior to Bn stimulation, we demonstrated that Bn-induced CREB phosphorylation was partially reduced, but not abolished (Fig. 3B), suggesting that the p38 MAPK pathway might be partially responsible for CREB phosphorylation. Furthermore, we showed that this inhibitor at 20 μM reduced p38 MAPK phosphorylation activated by Bn concomitantly with inhibition of CREB phosphorylation (Fig. 3B,C). In additional experiments, we showed that Bn did not induce p44/42 MAPK phosphoryl-

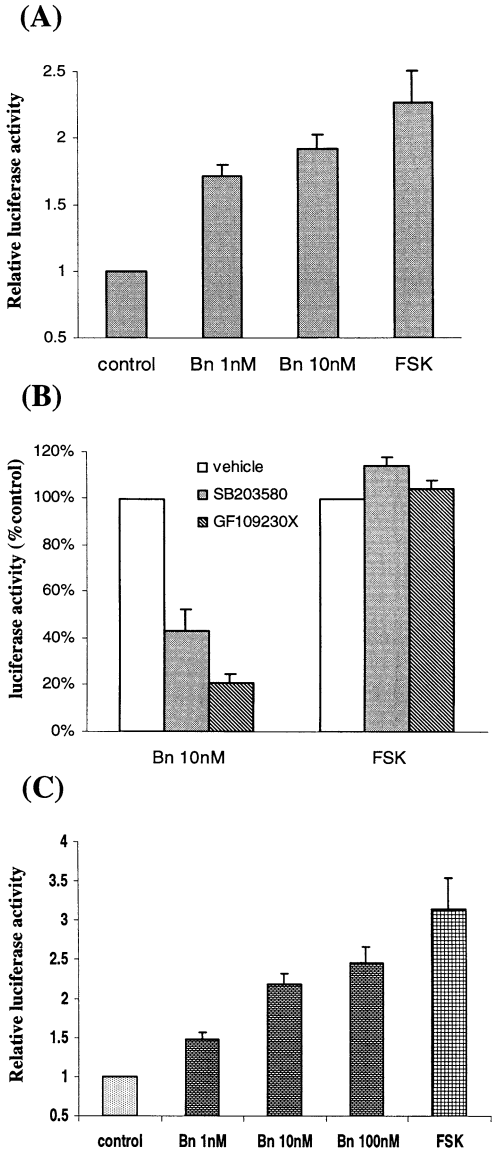


Fig. 4. Effects of Bn-dependent hGRP-R activation on CREB transcriptional activity and CRE-directed gene transcription. CREB transcriptional activation was assessed using a *trans*-reporter system in HuTu 80 cells. A: Cells were treated with vehicle, Bn (1 and 10 nM), and FSK (25 μM) for 16 h before cell lysis. Relative luciferase activity is expressed as mean value compared to activity measured in vehicle controls. Values are expressed as the mean (±S.E.M.) of five independent experiments, each performed in triplicate. B: Effects of GFX (2 μM) and SB 203580 (20 μM) on Bn (10 nM)- and FSK (25 μM)-induced CREB transactivation were expressed as the percentage of relative luciferase activity compared to control. Values are expressed as the mean (±S.E.M.) of four independent experiments, each performed in triplicate. C: The 4×SomCRE-Luc plasmid was transiently transfected into HuTu 80 cells to assess the effect of hGRP-R activation on CRE-directed gene transcription. Cells were treated with Bn and FSK (25 μM) for 16 h before cell lysis as indicated. Relative luciferase activity is expressed as mean value in each experiment compared to controls. Values are expressed as the mean (±S.E.M.) of five independent experiments, each performed in triplicate.

ation, and the MEK inhibitor U0216 (20 μM) did not affect Bn-induced CREB phosphorylation (data not shown). Moreover, inhibition of PKC by GFX (2 μM) did not reduce p38 MAPK phosphorylation (data not shown).

### 3.4. Human GRP-R activation mediates transcriptional activity of CREB as determined by a GAL4–CREB fusion protein

To examine whether hGRP-R activation can stimulate CREB-dependent transcriptional activity, we co-transfected a pFA2-CREB expression plasmid and a pFR-Luc reporter plasmid into HuTu 80 cells. The pFA2-CREB plasmid contains the human cytomegalovirus immediate early promoter to drive the constitutive expression of a fusion protein consisting of the activation domain of CREB fused with the yeast GAL4 DNA binding domain. Cells were treated with Bn and FSK for 16 h prior to cell harvest. Our results showed that both FSK and Bn resulted in increased CREB transactivation as measured by luciferase gene reporter assay in HuTu 80 cells (Fig. 4A). To further explore the role of PKC and the p38 MAPK pathway in Bn-induced CREB transactivation, we incubated the cells with GFX and SB 203580, respectively, prior to stimulation with Bn and FSK. GFX reduced Bn-induced CREB transactivation by almost 80% and SB 203580 diminished it by approximately 60%. In contrast, neither GFX nor SB 203580 resulted in any changes of FSK-induced CREB transactivation (Fig. 4B). These data provided further evidence that PKC as well as p38 MAPK activation are likely involved in Bn-dependent CREB phosphorylation and transactivation.

### 3.5. CRE-directed gene transcription is induced via hGRP-R activation

To determine whether Bn-induced CREB phosphorylation induced CRE-directed gene transcription, we tested a CRE reporter plasmid [8] in transient co-transfection experiments. Cells were transfected with a 4×SomCRE-Luc reporter plasmid along with the pCMVβ-gal plasmid. Stimulation with Bn for 16 h before cell harvest increased transcription of the luciferase reporter gene in a dose-dependent manner (Fig. 4C), suggesting that Bn-induced CREB phosphorylation and transactivation can enhance CRE-directed gene transcription in HuTu 80 cells.

## 4. Discussion

CREB, a 43 kDa leucine zipper transcription factor, is a main regulator of gene expression which mediates the activation of cAMP-responsive genes by binding as a dimer to a conserved cAMP-responsive element (CRE), characterized by the nucleotide octamer sequence TGACGTCA [6,9]. It was among the first transcription factors, which were shown to be regulated by phosphorylation. The transactivation domain of CREB consists of constitutive and an inducible domain that synergize in response to cAMP stimulation [10]. The KID is activated only when phosphorylated at $Ser^{133}$ [11]. Phosphorylation at this site potentiates activity of the KID by promoting recruitment of the co-activator paralogues CBP and p300 to the target promoter [12].

We showed that CREB phosphorylation at $Ser^{133}$ occurred in response to FSK, a stimulus known to produce CREB phosphorylation via a cAMP-dependent pathway, in duodenal cancer cells. In contrast, our studies are the first to demonstrate that Bn induced robust CREB phosphorylation sustained for at least 8 h. This effect is distinct from FSK-induced phosphorylation, which typically elicits a rapid, short-lived response. Our findings are specific for ligand-induced hGRP-R activation because Bn-induced CREB phosphorylation occurred even at sub-nanomolar concentrations, and specific receptor antagonists abolished the effect.

CREB is a substrate for many kinases other than PKA, including PKC, AKT, calcium-calmodulin-dependent kinases, mitogen/stress-activated kinase, and $pp90^{rsk}$ [6]. In this study, we determined further upstream signaling molecules involved in Bn-induced CREB phosphorylation. Bn has previously been shown to regulate transcription factor AP-1 activation through a PKC-dependent pathway in human gastric cancer SIIA cells [13]. However, it was not reported whether regulatory peptides induced PKC activation and subsequent CREB phosphorylation, which in turn could direct CRE-directed gene transcription. Our current study showed that Bn induced CREB phosphorylation, and its transcriptional activation is PKC- and partially p38 MAPK-dependent. Using immunoblot experiments, we demonstrated Bn-induced CREB phosphorylation was abolished in the presence of a PKC inhibitor, but not a PKA inhibitor. Furthermore, Bn also resulted in transcriptional CREB activation using a GAL4–CREB luciferase reporter system. Corresponding to our immunoblot data, pretreatment with GFX reduced Bn-induced CREB transcriptional activity, whereas it had no effect on FSK-induced CREB transcriptional activity.

In most circumstances, Bn was shown to raise cytosolic calcium levels, i.e. in pancreatic β cells Bn-induced insulin secretion was shown to be dependent on the increase of intracellular calcium [14]. However, in HuTu 80 cells, depletion of intracellular calcium by thapsigargin and BAPTA/AM did not prevent Bn-induced CREB phosphorylation, suggesting that Bn-induced CREB phosphorylation is calcium-independent.

Insulin-like growth factor I and nerve growth factor were shown to elicit p38 MAPK activation and result in CREB phosphorylation in PC12 cells, a pheochromocytoma cell line [15,16]. In rat pancreatic acini, Bn as well as cholecystokinin stimulated p38 MAPK activation [17]. Bn-induced AP-1 activation also required p38 MAPK activation in an intestinal epithelial cell line transfected with the murine GRP-R [18]. We show in this study that Bn induced sustained p38 MAPK phosphorylation for at least 4 h. Furthermore, our observations that SB 203580 reduced Bn-induced CREB phosphorylation and transactivation in immunoblot experiments and GAL4–CREB reporter system assays, respectively, suggest the possibility that p38 MAPK is partially responsible for Bn-induced CREB phosphorylation in HuTu 80 cells. SB 203580, a pyridinylimidazole compound, was initially found to be a selective inhibitor of p38 MAPK by competitive binding in the ATP pocket of p38 MAPK [19]. Although it is widely used as a specific inhibitor of p38 MAPK, it is also capable of inhibiting other protein kinases, such as p56 lck, c-src, and JNK2 β1 [20,21]. Therefore, our study cannot exclude the possibility that kinases other than p38 MAPK are involved in Bn-dependent CREB phosphorylation. We also found that inhibition of PKC by GFX did not abolish p38 MAPK phosphorylation, suggesting that Bn-induced CREB phosphorylation might be mediated by two independent pathways.

CREB-mediated transcription regulates diverse cellular

events including cellular metabolic regulation, cell survival and apoptosis, neuronal signaling and immune system function [6]. Previously, Bn was shown to activate CRE-mediated transcription in HIT-T15 cells, a hamster pancreatic β cell line [22]. However, the cellular events leading to Bn-induced CRE-mediated transcription were not further investigated. We demonstrate here that Bn had the ability to activate CRE-dependent transcription in gastrointestinal cancer cells with endogenously expressed, functional hGRP-R. Furthermore, our data indicate that Bn-induced CREB phosphorylation and transactivation might be responsible for transcription directed by the rat somatostatin CRE site. However, it still needs to be explored in future studies whether Bn-dependent CREB phosphorylation and regulation of downstream gene expression are similar or distinct compared with cAMP-dependent pathways. In this study, we used a human cancer cell line which possesses the native, functional GRP-R, and its ligand-specific activation was previously shown not to induce cell proliferation [23]. Therefore, one might speculate that hGRP-R-mediated CREB phosphorylation could be involved in gastrointestinal carcinogenesis through molecular mechanisms other than promoting cell proliferation directly. In this respect, it will be of considerable interest to further identify possible CREB-dependent target genes regulated through activation of hGRP-R.

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## References

[1] Kroog, G.S., Jensen, R.T. and Battey, J.F. (1995) Med. Res. Rev. 15, 389–417.
[2] Sunday, M.E., Kaplan, L.M., Motoyama, E., Chin, W.W. and Spindel, E.R. (1988) Lab. Invest. 59, 5–24.
[3] Carroll, R.E., Matkowskyj, K.A., Tretiakova, M.S., Battey, J.F. and Benya, R.V. (2000) Cell Growth Differ. 11, 385–393.
[4] Markwalder, R. and Reubi, J.C. (1999) Cancer Res. 59, 1152–1159.
[5] Jensen, J.A., Carroll, R.E. and Benya, R.V. (2001) Peptides 22, 689–699.
[6] Mayr, B. and Montminy, M. (2001) Nat. Rev. Mol. Cell Biol. 2, 599–609.
[7] Xiao, D., Wang, J., Hampton, L.L. and Weber, H.C. (2001) Gene 264, 95–103.
[8] Oetjen, E., Diedrich, T., Eggers, A., Eckert, B. and Knepel, W. (1994) J. Biol. Chem. 269, 27036–27044.
[9] Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. and Goodman, H.M. (1986) Nature 323, 353–356.
[10] Brindle, P., Linke, S. and Montminy, M. (1993) Nature 364, 821–824.
[11] Arias, J., Alberts, A.S., Brindle, P., Claret, F.X., Smeal, T., Karin, M., Feramisco, J. and Montminy, M. (1994) Nature 370, 226–229.
[12] Kwok, R.P. et al. (1994) Nature 370, 223–226.
[13] Kim, H.J., Evers, B.M., Litvak, D.A., Hellmich, M.R. and Townsend Jr., C.M. (2000) Am. J. Physiol. Cell Physiol. 279, C326–C334.
[14] Wollheim, C.B. and Pralong, W.F. (1990) Biochem. Soc. Trans. 18, 111–114.
[15] Xing, J., Kornhauser, J.M., Xia, Z., Thiele, E.A. and Greenberg, M.E. (1998) Mol. Cell Biol. 18, 1946–1955.
[16] Pugazhenthi, S., Miller, E., Sable, C., Young, P., Heidenreich, K.A., Boxer, L.M. and Reusch, J.E. (1999) J. Biol. Chem. 274, 27529–27535.
[17] Schafer, C., Ross, S.E., Bragado, M.J., Groblewski, G.E., Ernst, S.A. and Williams, J.A. (1998) J. Biol. Chem. 273, 24173–24180.
[18] Guo, Y.S., Hellmich, M.R., Wen, X.D. and Townsend Jr., C.M. (2001) J. Biol. Chem. 276, 22941–22947.
[19] LoGrasso, P.V., Frantz, B., Rolando, A.M., O'Keefe, S.J., Hermes, J.D. and O'Neill, E.A. (1997) Biochemistry 36, 10422–10427.
[20] Wadsworth, S.A. et al. (1999) J. Pharmacol. Exp. Ther. 291, 680–687.
[21] Lee, J.C., Kumar, S., Griswold, D.E., Underwood, D.C., Votta, B.J. and Adams, J.L. (2000) Immunopharmacology 47, 185–201.
[22] Eckert, B., Schwaninger, M. and Knepel, W. (1996) Endocrinology 137, 225–233.
[23] Williams, B.Y. and Schonbrunn, A. (1994) Cancer Res. 54, 818–824.